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Final Report

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Extended Portion

August 1974 - January 1975

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Contract No. NAS 9-13139

Early Detection of Disease Program:
Evaluation of the Cellular Immune Response

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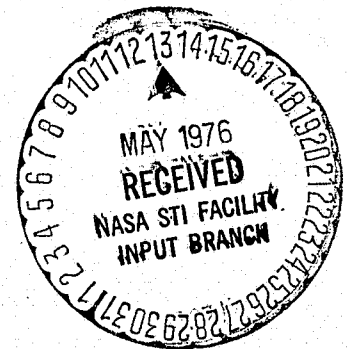
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"Extended Portion"

The following is a paper currently being submitted for consideration for publication by the Journal of Experimental Cell Research. This paper summarizes and presents in detail the work completed during the period of August 1974 - January 1975 for NAS 9 13139.

SCANNING ELECTRON MICROSCOPY OF NORMAL AND
MITOGEN-STIMULATED MOUSE LYMPHOID CELLS

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Running title: SEM of Mouse Lymphocytes

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SUMMARY

Surfaces of normal, cultured, and mitogen-stimulated mouse lymphoid cells have been examined by scanning electron microscopy (SEM). Lymphocytes with smooth, highly villous and intermediate surfaces were observed in cell suspensions from both spleens and thymuses of normal mice and from spleens of congenitally athymic (nude) mice. Several strain-specific surface features were noted. Most striking of these was the spine-like appearance of microvilli on C57Bl/6 lymphocytes. Although thymus cell suspensions contained somewhat more smooth cells than did spleen cell preparations, lymphocyte derivation could not be inferred from SEM examination. Studies of cells stimulated with agents mitogenic for thymus-derived lymphocytes (concanavalin A) or for bone marrow-derived lymphocytes (lipopolysaccharide) suggested instead that, in the mouse, development of a complex villous surface is a general concomitant of lymphocyte activation and transformation.

INTRODUCTION

Human lymphocytes of two distinct morphologic types have been described by Polliack et al. [1,2] and Lin et al. [3] utilizing scanning electron microscopy (SEM). One population was comprised of small lymphocytes with a relatively smooth surface. These cells, regarded as thymus-derived lymphocytes (T cells), formed rosettes with sheep erythrocytes or were obtained from sources of defined thymic origin. The other population had a complex villous surface, possessed easily demonstrable membrane-bound immunoglobulin, formed rosettes with complement-coated erythrocytes and were identified as bone-marrow derived lymphocytes (B cells).

We have studied the surface morphology of mouse lymphoid cells to ascertain whether lymphocytes from this species can be reliably classified by SEM examination. We have further investigated the effects of lymphocyte activation and transformation in response to stimulation with a T cell mitogen, concanavalin A (Con A) [4,5] and a B cell mitogen, E.coli lipopolysaccharide (LPS) [4,5].

MATERIALS AND METHODS

Mice: Male C57Bl/6, A/Tex and BALB/c mice were obtained from Texas Inbred Mice Co., Houston, Texas and the Department of Cell Biology, Baylor College of Medicine, and were maintained under standard conditions on laboratory chow and water ad libitum. Congenitally athymic nude mice (nu/nu) and their heterozygous litter mates (nu/+), backcrossed onto a BALB/c background, were bred in our laboratory and maintained on sterile water and laboratory chow in a laminar flow caging unit (Carworth, New York, N.Y.). Breeding pairs of nude mice were a gift of Dr. James D. Watson; derivation and details of backcrossing have been previously described [6]. Spleens and thymuses were removed from 12- to 16-week-old mice. Organs were teased in Hanks' balanced salt solution (HBSS) at 4°C and tissue debris was allowed to settle. Cells in suspension were divided into three groups and processed for tissue culture, scanning electron microscopy, and immunofluorescence.

Lymphocyte cultures: Cells were cultured as previously described [7] in RPMI 1640, supplemented with 2 mM glutamine, penicillin-streptomycin, and 5% fetal calf serum at 37°C in an atmosphere of 5% CO₂ in air. Con A (Nutritional Biochemicals Corp., Cleveland, O.) or LPS W from E. coli 0127:BB (Difco Laboratories, Detroit, Mich.) were added to cultures in graded concentrations at initiation. For assay of DNA synthesis tritiated thymidine (³H-TdR, New England Nuclear, Boston, Mass.), 1 µCi per culture,

was added to cultures during the final 16 hrs of the 48 hr incubation period. Cultures were harvested by washing with isotonic saline solution and aspirating onto glass fiber filters (MASH II, Microbiological Associates, Bethesda, Md.). Dried filters were placed in counting vials with scintillation solvent and radioactivity was assayed in a liquid scintillation spectrometer (Nuclear Chicago, Des Plaines, Ill.).

Scanning electron microscopy: $2-6 \times 10^6$ cells were washed once in 4°C HBSS. Cells were resuspended and fixed in 0.5% glutaraldehyde in phosphate buffered saline (PBS), pH 7.4, for 1 hr, and subsequently washed 3 times in cold PBS. After washing, lymphocytes were resuspended in 5 ml PBS and passed by gravity filtration through a PBS-moistened silver membrane of 0.8 μ m porosity (Flotronics, Spring House, Pa.). Cells were dehydrated with a graded series of ethyl alcohol (20-100%), followed by 50% amyl acetate in ethanol and 100% amyl acetate, and were critical-point dried according to the method of Anderson [8] in a Denton DCP-1 critical point drying apparatus (Denton Vacuum, Inc., Cherry Hill, N.J.). Membranes were coated with a thin layer of gold-palladium on a rotary stage of a vacuum evaporator (3 AM, Edwards High Vacuum, Ltd., Manor Royal, Crownby, England). Specimens were examined with a scanning electron microscope (ETEC Corp., Hayward, Calif.) with an accelerating voltage of 20 KV and a tilt angle of 30°. Micrographs were recorded on Polaroid type 52 P/N film (Polaroid Corp., Cambridge, Mass.).

Two hundred cells from coded samples were counted at random in the SEM, and classified as smooth, intermediate, or highly villous on the basis of surface morphology. Cells possessing a grossly pitted, deteriorating membrane or those lacking the characteristic spherical shape of lymphoid cells were omitted from the count. Cells were examined at 5000 x and representative photographs were taken of each sample.

Immunofluorescence: Cell suspensions were washed twice in 4°C PBS, resuspended, divided into three portions, and stained with one of the following fluorescein-conjugated goat antisera: anti-mouse IgM, anti-mouse IgG, or anti-mouse IgA (Meloy Laboratories, Springfield, Va.). After staining, samples were washed 3 times in 4°C PBS and examined with an Ortholux photomicroscope (E. Leitz, Inc., Rockleigh, N.J.) equipped with a ploem vertical illuminator and HBO 200 mercury burner. 200 cells were counted, alternating phase contrast with fluorescence illumination, and the number of fluorescent cells were noted.

RESULTS

Characterization of surfaces of lymphoid cells from normal and athymic mice: Splenic lymphocytes were classified by SEM in three catagories, according to the presence and density of villous projections from their surfaces (Table 1). One category, constituting 18-63% of the cells in most preparations, was relatively smooth, without villi or attachments seen on the surface of the cells (Fig. 1a). These cells were spherical,

3.0 to 3.3 μm in diameter, with a surface marked by slight undulations. On an occasional smooth cell one to ten small bump-like protrusions arose from the surface, however these projections were not regarded as villi since they were not seen on other cells that did have distinct villi. The second, and most prevalent, lymphocyte type (Fig. 1b) displayed a smooth or slightly ruffled surface with villous projections of about 0.5 μm . These were classified as intermediate cells. They comprised up to 78% of total lymphoid cells and were larger than the smooth cells (4.0 to 5.0 μm). A third cell type, also 4.0 to 5.0 μm in diameter, displayed numerous villi about 0.5 μm in length covering about 75% of the cellular surfaces (Fig. 1c). Usually 1-9% of normal splenic lymphocytes were of this third type.

Suspensions of normal mouse thymocytes could not regularly be differentiated by SEM from suspensions of splenic lymphocytes (Table I, Fig. 2a & 2b). A few (0-5%) highly villous cells were seen. Numbers of smooth cells were usually higher than observed in spleen. Most thymocytes, however, were of intermediate type, although somewhat less villous than spleen cells from the same animals.

When lymphoid cells from various mouse strains were compared, several differences were consistently observed. C57B1/6 spleen regularly contained more highly villous cells and lymphoid tissues from C3H/He mice contained relatively more smooth cells than were observed in spleens or thymuses of other strains (Table I).

Interestingly, T cell deficient spleens from congenitally athymic (nu/nu) mice contained more rather than fewer smooth cells than did normal BALB/c spleens. Several qualitative differences between strains were also observed. C57Bl/6 cells had villi which projected from the cellular surface, giving the appearance of "thorns" or "spines" (Fig.3a). Often the villi arose beside ridges and convolutions on the cell surface. In contrast villi on lymphocytes from nu/nu mice were somewhat shorter (0.1 - 0.2 μ m in length) (Fig.3b) than villi on lymphocytes from heterozygous (nu/+) or normal BALB/c mice (0.5 μ m in length) (Fig.1c). Villi on lymphocytes from C3H/He and A/Tex mice were similar to those on BALB/c cells, but were often finer in appearance.

Frequency of B cells in various suspensions was determined by staining cells for membrane-bound immunoglobulin with class-specific fluoresceinated antisera to mouse immunoglobulins (Table II). As expected, thymic preparations contained few lymphocytes with demonstrable membrane-bound immunoglobulin. T cell deficiency in nu/nu mice was reflected by a relative increase in percentage of B cells. No correlation was observed between numbers of villous lymphocytes and numbers of cells staining with fluoresceinated anti-immunoglobulins.

Effects of cell culture and mitogenic stimulation on surfaces of mouse spleen cells: Normal BALB/c and nu/nu spleen cells were cultured for various times from one minute to 48 hr at 37°C with Con A, or LPS, or in culture medium alone (Table III, Fig.4).

In the absence of a mitogenic stimulus there was no substantial change in the number of highly villous lymphocytes, although a small decrement in the number of smooth cells and a corresponding increase in intermediate cells was observed. Conversely, lymphocytes cultured with Con A or LPS exhibited marked alterations in surface morphology. BALB/c spleen cells were examined at 1 min, 5 min, 30 min, 60 min, 4 hrs, 24 hrs, and 48 hrs after addition of 1 $\mu\text{g/ml}$ Con A to culture media. Data summarized in Table III reveal that the most striking surface changes induced by Con A occurred after 24-48 hrs incubation, concomitant with peak DNA replication and with blast transformation by light microscopy. At these times, a marked increase in villous cells was observed (Fig.4a). These changes were not observed, however, in spleen cells from T cell-deficient mice cultured with Con A (Table III) or in normal BALB/c spleen cells cultured without a mitogen (Fig. 4b).

Several early morphologic changes were also observed in Con A-stimulated BALB/c spleen cell cultures. Within 1 min of Con A addition to cultures, the surfaces of smooth cells were altered from slightly ruffled to "blistered" with localized membrane aggregations of 0.08 μm to 0.1 μm diameter (Fig.4c). These cells morphologically resembled cells which had been cultured for 48 hr, but had not been stimulated by mitogens (Fig.4b). However these changes induced by conditions of culture occurred much more rapidly in the presence of Con A. After 5 min of

Con A stimulation, "blistering" was less apparent, however cell surfaces appeared increasingly ruffled and total cell diameters were decreased by 0.3 to 1.7 μm . Morphological features remained essentially constant at 1 and 4 hrs. At 24 and 49 hrs increasing numbers of larger (5.0 to 7.0 μm) and more villous cells were observed. Most villi of Con A-stimulated cells were 0.5 to 0.7 μm long, although occasional villi up to 1.0 μm in length were observed.

Replicative responses of lymphocyte populations were confirmed by assessing ^3H -TdR incorporation induced by T and B cell mitogens. Dose-response titrations for both Con A and LPS were studied and peak responses are illustrated (Table IV). As expected, both Con A and LPS induced a marked increase in DNA synthesis in normal spleen cells. ^3H -TdR incorporation by thymocytes was stimulated by Con A, but not by LPS and, conversely, nu/nu spleen cells responded to LPS, but not to Con A.

DISCUSSION

Lymphocytes have been classified by derivation as B and T cells utilizing numerous functional, biochemical and biophysical assays [9-11]. Recently SEM of human lymphoid cells has revealed differences in surface morphology which appeared to correlate with cell derivation [1,3]. T cells were described as smooth and B cells as highly villous; a variable number of cells were of intermediate surface morphology and uncertain derivation. Other studies of human and rabbit lymphocytes, however, have

shown that variations in cell preparation and fixation techniques may affect SEM appearance independent of derivation [12-14]. In fact, T cells from human peripheral blood which spontaneously form rosettes with sheep erythrocytes have been described as both smooth [2,3] and villous [2,15]. The data suggest that the process of rosette formation activates lymphocyte membranes and consequently induces development of microvilli in previously smooth cells [2,16].

We have also observed surface structural differences in suspensions of mouse thymocytes and spleen cells, with smooth, villous and intermediate lymphocytes. Cell derivation, however, was not well correlated with surface morphology since distribution of cell types in spleen and thymus was sufficiently similar to render suspensions indistinguishable on unbiased examination. Moreover, spleens from congenitally athymic mice possessed more rather than fewer smooth cells and only rare highly villous cells. It is unlikely that these observations reflect artifacts of technique induced by variation in temperature of fixation [12] or by cell settling before fixation [13,14] since cells were consistently fixed in suspension at 4°C.

Although surface morphology did not correlate well with cell derivation, strain-specific characteristics were identified. The most striking of these differences was the spine-like appearance of villi on C57Bl/6 lymphocytes. The functional significance of this qualitative strain-specific characteristic has not yet been determined. However, significant differences between

BALB/c and C57Bl/6 mice in blastogenic responses to mitogens have been described [17], and breeding experiments may demonstrate relationships, if any, between lymphocyte surface morphology and immunological reactivity.

The present data suggest that in the mouse alterations in lymphocyte surfaces observed by SEM are, in fact, concomitants of lymphocyte activation rather than of derivation. Our most striking observation was the marked increase in numbers of highly villous cells in suspensions prepared from mitogen-stimulated cultures. A T cell mitogen, Con A [4,5], and a B cell mitogen, LPS [4,5], effected similar membrane changes at times concordant with DNA replication and blast transformation in stimulated cultures. Similar changes were not observed during culture of non-stimulated normal cells, nor were they noted in cultures of Con A-treated nude spleen cells. Nevertheless a marked increase in villous lymphocytes was observed in nude spleen cell cultures stimulated by LPS. This observation is consistent with an hypothesis that in the mouse highly villous lymphocyte surfaces are SEM correlates of blast transformation rather than of cell derivation. The metabolic events during rosette formation between human lymphocytes and sheep erythrocytes may be substantially different from those of blast transformation. Nonetheless, the development of microvilli concomitantly with rosette formation, described by Polliack, et al. [2] and Lin and Wallack [16], suggests that this phenomenon might be a relatively general feature of lymphocyte membrane activation.

Surface alterations without villous transformation were also observed in spleen cell cultures within minutes of addition of Con A. It has become increasingly apparent that the early effects of Con A on both B and T cell membranes are various and complex. Thus, although capping and endocytosis of surface receptors for Con A may be observed [18], restriction of mobility of B cell surface immunoglobulin molecules is also well documented [19-21] and Con A-induced increases in membrane fluidity has been reported [22]. Further studies will be required to correlate the effects of Con A on surface macromolecules with the early structural consequences of Con A-binding reported here.

The present data, however, have led us to conclude that SEM examination is of little usefulness as an independent criterion of lymphocyte derivation in the mouse. Three observations were of particular importance in this conclusion: (1) we were unable to morphologically distinguish between cell suspensions from spleen and thymus on unbiased examination; (2) mitogen-activated lymphocytes, regardless of derivation, were highly villous; and (3) spleens of T cell-deficient mice contained large numbers of completely smooth cells. It is possible that species differences may account for disparities between our data and the rather striking correlation between surface morphology and derivation reported for human lymphocytes [1,3]. Nonetheless discrepancies, perhaps reflecting the differences in membrane stimulation, are reported among observers of T cell-sheep erythrocyte rosettes [2,3,14]. Clearly, future studies, regardless of species should establish

in addition to classification by traditional markers of lymphocyte derivation, the state of activation or metabolic characteristics of lymphoid cell populations described.

ACKNOWLEDGMENTS

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TABLE I

SEM Characterization of Mouse Lymphoid Cell Surfaces

Strain	Tissue	Cell Type*		
		% (Range)	Intermediate cells % (Range)	Villous cells % (Range)
BALB/c	Spleen	18 (11-26)	78 (70-81)	4 (1-5)
	Thymus	30 (25-34)	67 (65-70)	3 (1-5)
A/Tex	Spleen	25 (20-30)	73 (67-79)	2 (1-3)
	Thymus	30 (28-32)	66 (64-68)	4 (4-5)
C57Bl/6	Spleen	27 (27-28)	60 (55-66)	9 (7-11)
	Thymus	46 (46-46)	48 (45-51)	5 (3-8)
C3H/He	Spleen	38 (28-49)	59 (49-69)	2 (2-3)
	Thymus	63 (52-74)	34 (21-48)	3 (0-5)
BALB/c (nu/nu)	Spleen	34 (25-43)	65 (56-73)	1 (0-3)

* Percentages represent mean differential counts from 2-4 experiments per strain.
Two hundred cells from each tissue were examined for each experiment.

TABLE II

Lymphocytes with Membrane-bound Immunoglobulin
in Mouse Spleens and Thymuses

Strain	Tissue	Antiserum Specificity*		
		IgG %	IgA %	IgM %
C57Bl/6	Spleen	21	18	24
	Thymus	0	0	2
BALB/c	Spleen	20	24	19
	Thymus	2	1	1
BALB/c (nu/nu)	Spleen	40	11	20
BALB/c (nu/+)	Spleen	37	10	15

* Percentages represent mean differential counts from two experiments.

200 cells per experiment were examined for each antiserum and each tissue.

TABLE III

**Effects of Culturing with and without T and B cell Mitogens
on Surface Morphology of Mouse Spleen Cells**

Tissue	Mitogen	Time in Culture (hrs)	Smooth Cells* % (Range)	Intermediate Cells % (Range)	Villous Cells % (Range)
Normal BALB/c Spleen	None	0	24 (20-27)	72 (69-76)	4 (4-4)
	"	24	22 (21-24)	77 (76-79)	1 (0-1)
	"	48	15 (12-17)	80 (75-86)	5 (2-8)
	Con A	0	13 (11-14)	81 (81-82)	6 (5-7)
	"	1	23 (20-26)	71 (70-72)	6 (4-8)
	"	24	16 (16-17)	63 (62-63)	21 (20-23)
	"	48	15 (12-18)	37 (36-38)	48 (44-52)
	LPS	0	7 (3-11)	91 (87-96)	2 (1-2)
	"	1	8 (8-9)	90 (88-91)	2 (0-3)
	"	24	21 (16-26)	58 (54-61)	21 (20-23)
	"	48	15 (11-19)	39 (37-41)	46 (44-48)
nu/nu Spleen	None	0	31 (25-37)	68 (63-73)	1 (0-1)
	"	24	34 (27-40)	65 (58-72)	1 (1-2)
	"	48	34 (27-40)	65 (58-73)	1 (0-2)
	Con A	0	43 (30-33)	68 (67-70)	0 (0-0)
	"	24	30 (26-33)	67 (64-71)	3 (3-3)
	"	48	20 (10-30)	79 (68-89)	1 (1-2)
	LPS	0	31 (27-34)	67 (63-72)	2 (1-3)
	"	24	23 (16-30)	66 (64-69)	11 (6-15)
	"	48	17 (15-18)	54 (52-56)	29 (26-33)

*Percentages represent mean differential counts from two experiments.
200 cells per culture group were examined for each experiment.

TABLE IV

Response of Mouse Spleen Cells to B and T Cell Mitogens

Cell Source	Control* cpm	Mitogen			
		CPM A (1.0 μ g/ml)		LPS (5 μ g/ml)	
		cpm	E/C**	cpm	E/C
BALB/c Spleen	9,879	607,800	61.5	134,667	13.6
BALB/c Thymus	591	103,484	175.1	600	1.0
nu/nu Spleen	8,824	5,248	0.5	165,145	18.7

* Data represent mean counts per minute from triplicate cultures labeled at 32 h with 1 μ Ci 3 H-TdR and harvested at 48 h.

** E/C = cpm in mitogen-stimulated cultures divided by cpm in control cultures.

Figure Legends

- Fig. 1.** Spectrum of lymphocyte morphology from a normal BALB/c spleen. Fig.1a illustrates a smooth lymphocyte with only minor surface irregularities but without microvilli. x 13,738. Fig.1b shows a typical cell of intermediate surface morphology. x 13,629. Cells of this type exhibited a smooth or slightly ruffled surface with microvilli. Fig.1c illustrates a highly villous cell. x 6,693. Microvilli covered most of the surface of cells in this category.
- Fig. 2.** Spectrum of lymphocytes seen in thymus (2a) and spleen (2b) cell preparations. Most lymphocytes in both fields are of intermediate type; determination of tissue origin on unbiased examination was not regularly possible. Fig. 2a, x 5520. Fig. 2b, x 6052.
- Fig. 3.** Morphological differences in lymphocyte microvilli. Fig. 3a shows a C57Bl/6 lymphocyte. The "thorny" or "spine-like" appearance of villi extending from a broad base of the cell surface was characteristic of the strain x 20,428. Fig.3b illustrates a spleen cell from a congenitally athymic (nude) mouse. x 21,295. Microvilli on cells from these animals were somewhat shorter than those observed on lymphocytes from normal BALB/c mice (Fig. 1c).

Fig. 4. Effects of stimulation with mitogens of lymphocyte surface morphology. Fig. 4a illustrates BALB/c spleen cells after culturing for 48 h with Con 1, $1 \mu\text{g/ml}$. Most cells from Con A-stimulated cultures exhibited numerous microvilli. $\times 4,850$. Fig. 4b shows lymphocytes cultured for 48 h without addition of mitogens. $\times 6,100$. Frequency of microvilli was unchanged by 48 h culture without mitogen, however many smooth cells displayed a "ruffled" appearance after this time. Fig. 4c illustrates a qualitative change in lymphocyte surface morphology observed after a 1 min exposure to Con A. This treatment markedly accelerated the process of "ruffling" or "blistering" observed as a consequence of prolonged culture without mitogen. $\times 7,870$.